HEPATITIS

Resistance to adefovir dipivoxil in lamivudine resistant chronic hepatitis B patients treated with adefovir dipivoxil

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Background: Adefovir dipivoxil (ADV) is a potent nucleotide analogue against both the wild-type and lamivudine (LMV) resistant hepatitis B virus (HBV). The cumulative incidence of ADV resistant mutations in the nucleoside/-tide treatment naïve chronic hepatitis B patient (CHB) at weeks 48, 96, and 144 was 0, 0.8–3%, and ~5.9%, respectively.
Aims: The aim of this study was to characterise the genotypic and phenotypic mutation profiles to ADV in 67 LMV resistant CHB patients who were treated with ADV.
Methods: Serum HBV DNA was quantified by real time polymerase chain reaction. The ADV mutant was detected using matrix assisted laser desorption/ionisation time of flight mass spectrometry based genotyping assays, termed restriction fragment mass polymorphism (RFMP).
Results: RFMP analysis revealed that a total of 11 amino acid substitutions developed in the rt domain of the HBV polymerase in nine patients. The cumulative incidence of genotypic ADV resistance at months 12 and 24 was 6.4% and 25.4%, respectively. The rtA181V, rtN236T, and rtA181T mutations were detected in five, four, and two of the 67 patients at treatment months 12–17, 3–19, and 7–20, respectively. Serial quantification of serum HBV DNA revealed that two patients with the rtA181V mutation, with or without the rtN236T mutation, and one patient with the rtA181T mutation displayed HBV DNA rebound.
Conclusion: Emergence of the ADV mutation in LMV resistant patients who are treated with ADV appeared to present earlier and more frequently than was reported in previous studies on nucleoside/-tide treatment naïve patients.

One of the problems of prolonged lamivudine (LMV) treatment is that a LMV resistant mutant can emerge, and this can lead to exacerbation of hepatitis or liver failure and so reduce the advantage of LMV.[1 2]

The benefit of adefovir dipivoxil (ADV) therapy compared with LMV for chronic hepatitis B (CHB) patients has been the delayed and infrequent selection of drug resistant viruses.[3–6] The cumulative incidence of an ADV resistant mutation emerging in nucleoside treatment naïve CHB patients at 48, 96, and 144 weeks was 0%, 0.8–3%, and ~5.9%, respectively.[7–9] Among the various conserved amino acid substitutions sites, including rtS119A, rtH133L, rtV214A, and rtH234Q, the amino acid substitution at the rtN236T site is known to confer marked reduction in susceptibility to ADV, and this causes biochemical and hepatitis B virus (HBV) DNA rebound in selected patients.[10 11]

The number of LMV resistant mutant patients who might be a potential candidate for ADV treatment is growing, and this calls for an assessment of the resistance profiles for the ADV mutations in a large number of LMV resistant CHB patients who are being treated with ADV.

The aims of our study are to analyse the A181V/T and N236T mutations of the HBV polymerase in LMV resistant CHB patients treated with ADV, and also to characterise the clinical consequences of ADV mutant selection.

MATERIALS AND METHODS

Patients

Data were collected retrospectively from 106 LMV resistant CHB patients who started ADV treatments from December 2002 to December 2004 at the Liver Clinic Centre of the Korea University Guro-Hospital in Seoul, Korea. Sera were collected from patients every two to three months during treatment and kept at −20℃ until the mutation analyses were performed. Patients who were treated with ADV for less than three months were excluded. Finally, a total of 67 patients, whose sera was available, were included in our study. All 67 patients were treated with ADV alone with (n = 16, 1–3 months) or without (n = 51) initial LMV. All of the serum samples were found to be genotype C by the INNO-LiPA HBV genotyping line probe assay (Innogentics NV, Ghent, Belgium). Informed consent was obtained from each patient and the experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected by a priori approval of the Korea University Guro Hospital human research committee.

Biochemical and virological monitoring

Routine complete blood count and a liver biochemical study, including aspartate aminotransferase, alanine aminotransferase, total bilirubin, albumin, prothrombin time, blood urea nitrogen, and creatinine test were performed for each patient. Hepatitis B surface antigen and antibody to the hepatitis B surface antigen were measured using commercially available radioimmunoassay kits (Abbot Laboratories, North Chicago,

Abbreviations: ADV, adefovir dipivoxil; LMV, lamivudine; CHB, chronic hepatitis B; HBV, hepatitis B virus; LC, liver cirrhosis; LC-c, compensated liver cirrhosis; LC-d, decompensated liver cirrhosis; HBeAg, hepatitis B e antigen; PCR, polymerase chain reaction; RFMP, restriction fragment mass polymorphism; RT, reverse transcription

Table 1 Expected masses of oligonucleotides resulting from restriction enzyme digestion of polymerase chain reaction products

Codon 180–181	Expected fragment mass (Da)			
	12 mer	13 mer	14 mer	15 mer
L-A (CTG GCT)	CAGGAGAAACGG 3808.4	CCAGGAGAAACGG 4097.6	CCGTTTCTCCTGGC 4261.8	CCGTTTCTCCTGGCT 4566.0
M-A (ATG GCT)	CATGAGAAACGG 3783.4	CCATGAGAAACGG 4072.6	CCGTTTCTCATGGC 4285.8	CCGTTTCTCATGGCT 4590.0
L-T (CTG ACT)	CAGGAGAAACGG 3808.4	TCAGGAGAAACGG 4112.6	CCGTTTCTCCTGAC 4245.8	CCGTTTCTCCTGACT 4550.0
M-T (ATG ACT)	CATGAGAAACGG 3783.4	TCATGAGAAACGG 4087.6	CCGTTTCTCATGAC 4269.8	CCGTTTCTCATGACT 4574.0
L-V (CTG GTT)	CAGGAGAAACGG 3808.4	CCAGGAGAAACGG 4097.6	CCGTTTCTCCTGGT 4276.8	CCGTTTCTCCTGGTT 4581.0
M-V (ATG GTT)	CATGAGAAACGG 3783.4	CCATGAGAAACGG 4072.6	CCGTTTCTCATGGT 4300.8	CCGTTTCTCATGGTT 4605.0

Sequences corresponding to the codon of interest in the oligonucleotides are underlined.

Illinois, USA). Hepatitis B e antigen (HBeAg) and antibody to the hepatitis B e antigen were measured by radioimmunoassay kits (BJ INEM.Co., Beijing, China). Tests for biochemical liver function and viral replication were assessed every two to three months during ADV treatment.

Definition

Genotypic mutation was defined as follows: HBV polymerase mutation analysis using mass spectrometric analysis of the oligonucleotide fragment, termed restriction fragment mass polymorphism (RFMP) or sequencing analysis, revealed the conserved site mutations of the reverse transcriptase domains A through E, including the rtA181 or rtN236 mutations. HBV DNA rebound was defined as follows: an increase in serum HBV DNA of greater than or equal to 1 $\log_{10}$ copies/ml from the ADV treatment nadir. Decompensated cirrhosis was defined as those patients with liver cirrhosis who showed one of the following complications: ascites, variceal bleeding, or hepatic encephalopathy, or had a score greater than or equal to a Child-Turcott-Pugh score of 9.

HBV DNA quantification by real time polymerase chain reaction (PCR)

Quantitative analysis of serum HBV DNA was performed retrospectively from stored serum samples. For real time reverse transcription (RT)-PCR quantification, viral DNA was extracted using a Qiagen Blood Kit (Qiagen, Chatworth, California, USA) according to the manufacturer's instructions. Real time PCR was performed with an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, California, USA). The amplification reaction was performed in 25 µl of reaction mixture containing 1× TaqMan buffer (PE Biosystems, Foster City, California, USA), 300 nM of each primer, 250 nM TaqMan probe (PE Biosystems), 0.5 U of AmpErase uracil N-glycosylase, and 1.5 U of AmpliTaq Gold DNA polymerase (PE Biosystems). Amplification conditions were 50℃ for two minutes, 95℃ for 10 minutes, and 45 cycles of 95℃ for 15 seconds and 62℃ for one minute. The respective sequences of forward primer, reverse primer, and TaqMan probe were as follows: 5′-CTC CCC GTC TGT KCC TTC TCA TC-3′ (HBVRT1F, nucleotides 1542-1564; K = G or T), 5′-GGC GTT CAC GGT GGT CTC CAT GC-3′ (HBVRT1R, nucleotides 1625-1603), and 5′ FAM-CCG TGT GCA CTT CGC TTC ACC TCT GC-TAMRA 3′ (HBV1TAQ, nucleotides 1575-1600). Nucleotide sequence positions were numbered according to Ono and colleagues.[12] For preparation of an external standard containing non-infectious material, two tandem repeats of HBV genomic DNA (GenBank X14193) were cloned into vector pUC119 and transformed into HB101

Table 2 Expected masses of oligonucleotides resulting from restriction enzyme digestion of polymerase chain reaction products

Codon 236	Expected fragment mass (Da)					
	9 mer	11 mer	11 mer	13 mer	12 mer	14 mer
N (AAC)	GGTTtAAAT 2832.8	ATTTaAACCCT 3355.2	AGGGTTtAAAT 3475.2	ATTTaAACCCTAA 3981.6	TAGGGTTtAAAT 3779.4	ATTTaAACCCTAAT 4285.8
N (AAT)	GATTtAAAT 2816.8	ATTTaAATCCT 3370.2	AGGATTtAAAT 3459.2	ATTTaAATCCTAA 3996.6	TAGGATTtAAAT 3763.4	ATTTaAATCCTAAT 4300.8
T (ACC)	GGGTtAAAT 2857.8	ATTTaACCCCT 3316.2	AGGGGTtAAAT 3516.2	ATTTaACCCCTAA 3942.6	TAGGGGTtAAAT 3820.4	ATTTaACCCCTAAT 4246.8
T (ACT)	GAGTtAAAT 2841.8	ATTTaACTCCT 3331.2	AGGAGTtAAAT 3500.2	ATTTaACTCCTAA 3957.6	TAGGAGTtAAAT 3804.4	ATTTaACTCCTAAT 4261.8
N (AAC)*	GGTTcAAAT 2817.8	ATTTgAACCCT 3371.2	AGGGTTcAAAT 3460.2	ATTTgAACCCTAA 3997.6	TAGGGTTcAAAT 3764.4	ATTTgAACCCTAAT 4301.8
N (AAT)*	GATTcAAAT 2801.8	ATTTgAATCCT 3386.2	AGGATTcAAAT 3444.2	ATTTgAATCCTAA 4012.6	TAGGATTcAAAT 3748.4	ATTTgAATCCTAAT 4316.8
T (ACC)*	GGGTcAAAT 2842.8	ATTTgACCCCT 3332.2	AGGGGTcAAAT 3501.2	ATTTgACCCCTAA 3958.6	TAGGGGTcAAAT 3805.4	ATTTgACCCCTAAT 4262.8
T (ACT)*	GAGTcAAAT 2826.8	ATTTgACTCCT 3347.2	AGGAGTcAAAT 3485.2	ATTTgACTCCTAA 3973.6	TAGGAGTcAAAT 3789.4	ATTTgACTCCTAAT 4277.8

Sequences corresponding to the codon of interest in the oligonucleotides are underlined.
A to G polymorphism at nucleotide position 834 is indicated by a lower case letter; most genotypes contain A base while parts of the hepatitis B virus genotypes C, F, and H strains* contain G base at the position. See supplementary table 1 for details (supplementary table 1 can be viewed on the *Gut* website at http://www.gutjnl.com/supplemental).

Table 3 Baseline characteristics of 67 lamivudine resistant chronic hepatitis B patients treated with adefovir dipivoxil

Variable	With ADV mutation (n = 9)	Without ADV mutation (n = 58)	Total (n = 67)	p Value
Age†	48.5 (1.9)	47.4 (1.16)	47.6 (1.0)	NS
Sex (M/F)	8/1	45/13	53/14	NS
HBV DNA (log10 copies/ml)*†	7.39 (0.26)	7.32 (0.14)	7.3 (1.04)	NS
ALT (IU/l)†	158.4 (33.9)	299.0 (38.6)	280 (34)	NS
AST (IU/l)†	116.3 (17.8)	212.7 (32.6)	199.7 (38.6)	NS
Total bilirubin(mg/dl)†	2.0 (0.46)	1.58 (0.15)	1.64 (1.19)	NS
Albumin (g/l)†	3.38 (0.25)	3.90 (0.08)	3.80 (0.65)	0.024
HBeAg (+)/(−)	7/2	44/14	51/16	NS
Duration of ADV treatment (months)†	17.3 (0.8)	15.5 (0.5)	15.6 (0.49)	NS
Pretreatment YIDD/YVDD/mixed	5/0/3	27/12/18	32/12/21	NS
L180M (+/−/mixed)	3/0/5	24/14/20	27/14/25	NS
CHB/LC-c/LC-d	1/5/3	38/16/4	39/21/7	0.015

*Viral loads are determined by real time polymerase chain reaction.
†Values are represented as mean (SEM).
HBV, hepatitis B virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; ADV, adefovir dipivoxil; CHB, chronic hepatitis B; LC-c, compensated liver cirrhosis; LC-d, decompensated liver cirrhosis.

Escherichia coli bacterial strain. Plasmid DNA was purified by ultracentrifugation on CsCl gradients. The standard plasmid DNAs were calibrated against the World Health Organization International Standard for HBV DNA (NIBSC, Potters Bar, UK): 1 IU/ml was converted into 5 copies/ml according to Saldanha and colleagues.[13] DNA concentrations of samples were calculated from standard curve equation (Ct = slope × log copy + intercept). Linear dynamic range of detection was found to be 366–3.66×10^{11}copies/ml ($r^2 = 0.998$).

ADV and LMV resistant mutation analysis by mass spectrometric analysis of the oligonucleotide fragment (RFMP)

Viral DNA (2 µl) was used for the PCR reaction. For RFMP genotyping, PCR was performed in an 18 µl reaction mixture containing 20 mM Tris HCl (pH 8.4), 50 mM KCl, 0.2 mM of each dNTP, 10 pmol of each primer, and 0.4 units of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). Amplification conditions included initial denaturation at 94℃ for two minutes followed by 35 cycles of denaturation at 94℃ for 30 seconds, annealing at 55℃ for 30 seconds, and extension at 72℃ for 30 seconds. The sequences for the forward and reverse primers used in the PCR are described in the appendix. Sequences with small letters were engineered to insert *Mme*I and *Hae*III, and *Mme*I and *Ssp*I restriction recognition sites in amplicons for rtA181V/T and rtN236T, respectively. Restriction enzyme digestion of PCR products was performed by mixing the PCR mixture with 10 µl of reaction buffer containing 5 mM potassium acetate, 2 mM Tris acetate, 1 mM magnesium acetate, 1 mM dithiothreitol and 1.5 units of *Mme*I (NEB, Boston, Massachusetts, USA). The reaction mixtures were incubated at 37℃ for two hours and further incubated at 37℃ for two hours with *Hae*III or *Ssp*I (NEB) for analysis of rtA181V/T or rtN236T, respectively. Mass spectra were acquired on a linear MALDI-TOF MS (Bruker Daltonics Biflex IV, Billerica, Massachusetts, USA) workstation, as previously described.[14] Expected mass for each codon is shown in tables 1 and 2.

Sequencing analysis of the entire HBV polymerase

To amplify the HBV polymerase gene coding for amino acids 380–615 for sequencing analysis, PCR was performed using primers BF105 (5′- TCC TGC TGC TAT GCC TCA TC-3′, nucleotide number 411-430) and BR112 (5′-TTC CGT CCA CAT ATC CCA TGA AGT TAA GGG A-3′, nucleotide number 895-865), as described by Chayama and colleagues,[2] at baseline, week 48, and week 96 of treatment. For confirmation of viral quasispecies, we cloned the PCR amplicons into plasmids (pCR-Script Amp cloning vector; Stratagene, La Jolla, California, USA). Ten clones were initially sequenced for each sample; additional clones were sequenced when the presence of a mixed population could not be demonstrated. Sequence analysis was performed by ABI PRISM 310 Genetic Analyzer (Applied Biosystems, New York, New York, USA).

Statistical analysis

Values are expressed as means (SEM) for experiments that compared patients with or without the ADV mutation. The proportion of patients who had predefined laboratory abnormalities was recorded, and between patients with or without mutant comparisons were performed using χ^2 tests of association. The Kaplan-Meier method was used to estimate the time to development of genotypic and phenotypic resistance to ADV. A p value <0.05 was considered statistically significant.

RESULTS

Demographic characteristics

Table 3 summarises the baseline characteristics and demographics for the 67 LMV resistant patients who were treated with ADV. Thirty nine of 67 patients (60%) had CHB. The remaining 28 of the 67 patients (40%) had either compensated (LC-c, n = 21) or decompensated (LC-d, n = 7) liver cirrhosis (LC). Mean HBV DNA level, presented as $\log_{10}$ copies/ml, was 7.3. Mean duration of treatment was 15.6 months.

Genotypic analysis of the ADV mutation in rtA181 and rtN236

During the course of the ADV treatment, a total of nine LMV resistant patients who were treated with ADV developed the amino acid substitution at the conserved sites in the HBV-RT domain (table 4). Representative RFMP analyses of the rtA181V and rtN236T mutations are shown in fig 1. We did not see any deviations from the predicted mass patterns in our samples that could be due to genotype differences or the existence of potential polymorphisms. RFMP analysis revealed that nine patients had developed a total of 11 amino acid substitutions on the rt domain of HBV polymerase during the course of ADV treatment. The rtA181V, rtN236T, and rtA181T mutations were detected in five, four, and two of the 67 patients at treatment months 12–17, 3–19, and 7–20, respectively. Two of the five rtA181V patients were

Table 4 Clinical characteristics of the nine adefovir dipivoxil (ADV) resistant patients

Patient No	Age (y)	Sex	Disease	HBeAg/ab	ALT (IU/l)	HBV DNA (log 10 copies/ml)	Type of ADV mutation (appeared at treatment month)
1	50	M	LC-c	+/−	139	6.45	rtA181V (16)
2	57	M	LC-d	+/−	80	8.59	rtA181V (13), rtN236T (19)
3	45	M	LC-d	+/−	51	9.90	rtA181T (20)
4	57	F	LC-d	−/+	157	7.31	rtA181T (7)
5	46	M	CHB	+/−	231	8.18	rtN236T (10), rtA181V (13)
6	40	M	LC-c	+/−	338	8.59	rtA181V (12)
7	48	M	LC-d	+/−	39	6.19	rtN236T (3)
8	44	M	LC-c	−/+	183	7.31	rtN236T (16)
9	50	M	LC-c	+/−	139	7.13	rtA181V (17)

HBV, hepatitis B virus; ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; CHB, chronic hepatitis B; LC-c, compensated liver cirrhosis; LC-d, decompensated liver cirrhosis.

simultaneously carrying the rtN236T mutation (table 4). Comparison of baseline characteristics of the patients without (n = 58) or with (n = 9) the ADV mutation did not revealed any statistically significant differences in baseline ALT, treatment duration, presence or absence of HBeAg, or baseline serum HBV DNA levels (table 3). In contrast to the patient group without the ADV mutation, the patients with the ADV mutation were either LC-c (n = 5) or LC-d (n = 3).

Cumulative incidence of genotypic mutation and HBV DNA rebound in LMV resistant CHB patients treated with ADV

The cumulative incidence of the genotypic mutation and HBV DNA rebound in LMV resistant CHB patients treated with ADV is shown in fig 2. At treatment months 12 and 24, four and 11 cases of genotypic ADV mutation developed in LMV resistant CHB patients who were treated with ADV, respectively. The cumulative incidence of genotypic ADV resistance at months 12 and 24 were 6.4% and 25.4%, respectively (fig 2A). HBV DNA rebound was observed in six of 67 patients. Three patients carried genotypic mutations but the other three patients did not (fig 2B).

Clinical courses for patients with the ADV resistant mutation

During the course of ADV treatment, three of nine ADV mutant patients developed HBV DNA rebound. The serial serum HBV DNA quantification analysis and ALT levels for these patients are shown in fig 3.

Patient No 1 (fig 3A), who had LC-c with YIDD and had the L180M mutation, showed a baseline HBV DNA level of 6.45 $\log_{10}$ copies/ml. This patient's baseline serum ALT was 139 IU/l which declined to the normal range at treatment month 16. The treatment nadir of HBV DNA was 4.04 $\log_{10}$ copies/ml at treatment month 13. After 19 months of ADV treatment, HBV DNA increased to a maximum value of 5.08

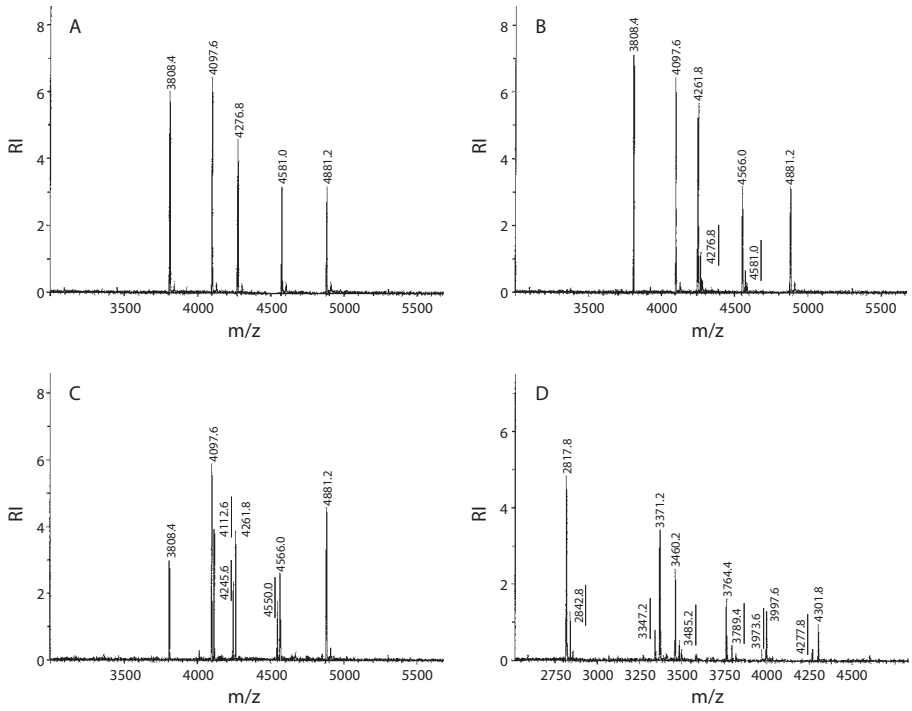

Figure 1 Representative matrix assisted laser desorption/ionisation time of flight spectra showing rtA181T/V and rtN236T mutations in lamivudine (LMV) resistant chronic hepatitis B patients treated with adefovir dipivoxil (ADV). (A) Mass spectrum of rt181V; (B) mass spectrum of mixed rt181A and rt181V; (C) mass spectrum of mixed rt181A and rt181T; and (D) mass spectrum of mixed rt236N and rt236T. RI and m/z represent relative peak intensity and mass to charge ratio, respectively. An internal mass control with a mass of 4881.4 is shown in A–C panels. Mass peak corresponding to minor amounts of mutant virus in the mixed population are underlined.

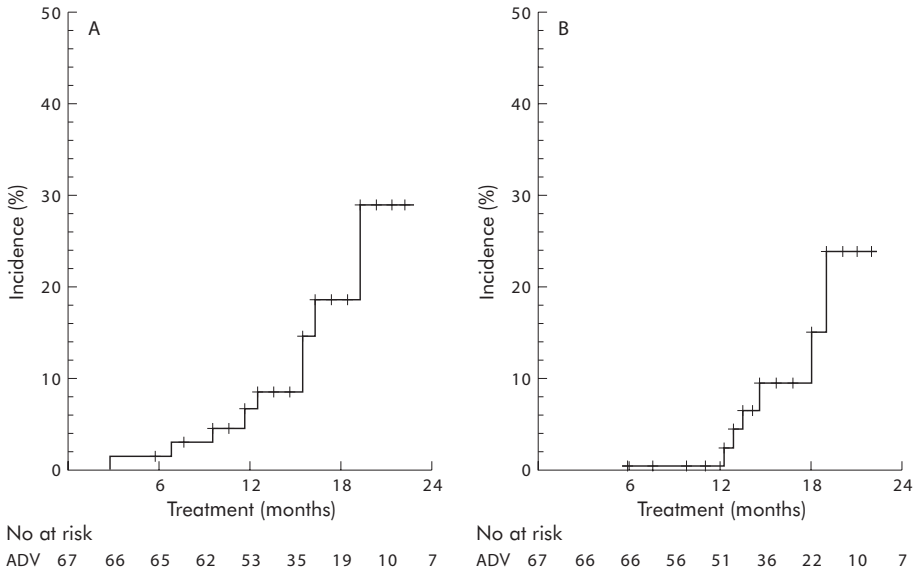

Figure 2 Cumulative incidence of genotypic mutation and hepatitis B virus (HBV) DNA rebound in adefovir dipivoxil (ADV) resistant chronic hepatitis B (CHB) patients. Cumulative incidence of genotypic mutation (A) and HBV DNA rebound (B) in lamivudine resistant CHB patients treated with ADV.

log_{10} copies/ml with a normal ALT. Retrospective analysis of the ADV mutation revealed the rtA181V mutation at treatment month 16.

Patient No 2 (fig 3B) had LC-d with YIDD, YVDD, and this patient had the L180M mutation. Baseline HBV DNA was 8.59 log_{10} copies/ml and ALT was 80 IU/l. After three months of ADV therapy, HBV DNA declined to the treatment nadir level of 4.6 log_{10} copies/ml. After 16 months of ADV treatment, HBV DNA rebounded to 7.7 log_{10} copies/ml. Serum ALT declined to the normal range at treatment month 10 and it increased up to 64 IU/l at treatment month 14. Thereafter, ALT declined to the normal range at treatment month 16 and it remained unchanged. Baseline serum bilirubin was 2.7 mg/dl and then increased to its maximum level of 5.6 mg/dl at treatment month 3; thereafter, bilirubin decreased to 1.1 mg/dl at treatment month 13. HBeAg loss occurred at treatment month 10 but this reverted to positive at treatment month 14. After 16 months of ADV therapy, LMV was then added and HBV DNA and serum ALT started to decrease. In the retrospective analysis, rtA181V and rtN236T mutations were detected at treatment months 13 and 19, respectively.

Patient No 3 (fig 3C) had LC-d. Baseline HBV DNA was 9.90 log_{10} copies/ml and serum ALT was 51 IU/l. HBV DNA declined to the treatment nadir level of 5.3 log_{10} copies/ml at treatment month 15. After 20 months of ADV treatment, HBV DNA level increased to a maximum level of 6.8 log_{10} copies/ml. Serum ALT decreased to the normal range at treatment month 6 and remained unchanged. Baseline serum bilirubin was 5.1 mg/dl which decreased to 2.5 mg/dl during the course of ADV therapy. The retrospective analysis of the ADV mutation revealed the rtA181T mutation at treatment month 20.

Six of the nine ADV mutant patients did not develop HBV DNA rebound. HBV DNA quantification using real time PCR showed a 2–4 log_{10} copies/ml reduction in HBV DNA after initiation of ADV therapy. HBV DNA and serum ALT levels remained unchanged even after development of the rtA181V or rtN236 mutations at treatment months 12–17 and 3–16, respectively.

Comparison of HBV reduction in patients with or without the ADV mutation

To characterise patients belonging to the group with or without the ADV mutation, the magnitude of the HBV DNA reductions at treatment months 3, 6, 9, and 12 were compared. As shown in fig 4, the median reduction, as represented by log_{10} copies/ml at treatment months 3 and 6

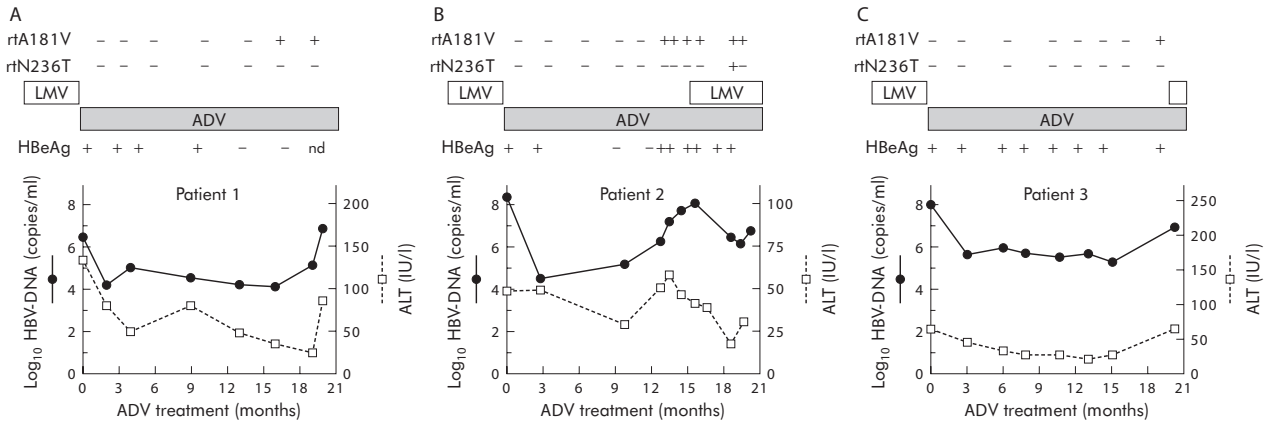

Figure 3 Clinical courses for patients with the adefovir dipivoxil (ADV) resistant mutation. Serial quantification of hepatitis B virus (HBV) DNA and alanine aminotransferase (ALT) in patients with the ADV resistant mutation. During the course of ADV treatment, three of the nine patients with the ADV resistant mutant (patient No 1 (A), No 2 (B), and No 3 (C)) developed HBV DNA rebound. LMV, lamivudine; HBeAg, hepatitis B e antigen.

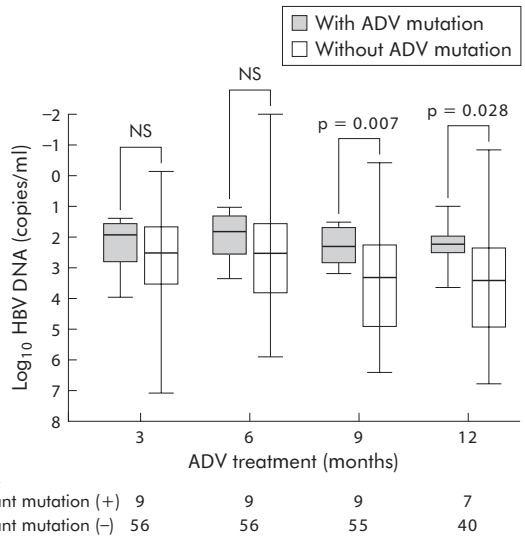

Figure 4 Comparison of hepatitis B virus (HBV) reduction in patients with or without the adefovir dipivoxil (ADV) resistant mutation. Median HBV DNA reduction at ADV treatment months 3, 6, 9, and 12 from baseline in patients with or without the ADV mutation. Horizontal bars represent median values. Upper and lower ends of each bar are quartile values.

were 2.2 versus 2.8 (NS) and 1.9 versus 2.8 (NS) in patients with or without the mutation, respectively. At treatment months 9 and 12, the amount of the HBV DNA reduction was 2.3 versus 3.5 ($p = 0.007$) and 2.1 versus 3.5 ($p = 0.028$) in patients with or without the mutation, respectively.

Proportion of the ADV mutant in the nine patients with the genotypic mutation

Using RFMP analysis, the proportion of the ADV mutant in the total viral population was calculated. For patients with no HBV DNA rebound, the mutant virus comprised less than 33% of the total viral population. In contrast with patients with no HBV rebound, the two rtA181V patients with HBV DNA rebound showed a mutant proportion of over 50–100% of the total viral population. In one patient with the rtA181T mutation, HBV DNA rebound was observed at the stage when the mutant comprised 40% of the total population (table 5).

Sequence analysis of ADV mutation

Existence of the conserved site mutations shown in RFMP was further verified by direct or clonal sequencing of PCR products spanning sequences encoding the polymerase domain. Sequence analysis showed that all samples were genotype C, and A to G polymorphism at nucleotide position 834 and C to T variation at nucleotide position 837 were not present. Examination of the adefovir resistance mutations suggested by Bartholomeusz and colleagues[15] from molecular modelling of the polymerase at the D and A domains (rtP237H, rtN238T/D, rtV84M, rtS85A) and the C-D inter-domain (rtV214A, rtQ215S) showed no additional mutations.

DISCUSSION

ADV is a nucleotide analogue of adenosine monophosphate. ADV has recently been approved for treatment, and it is a potent antiviral drug against both the wild-type and LMV resistant HBV.[3–7 16–19] In contrast with LMV therapy, ADV therapy is associated with delayed and infrequent selection of drug resistant viruses in the nucleos(-t)ide naïve patient.[7 8 20]

Angus and colleagues[10] reported the first case of a novel HBV polymerase N236T mutation against ADV in a nucleotide naïve liver cirrhosis patient at treatment week 80.

Table 5 Relative abundance of the adefovir dipivoxil (ADV) mutant in patients with or without hepatitis B virus (HBV) DNA rebound

HBV DNA rebound	Patient No	rtA181V	rtA181T (mutant percentage)	rtN236T
Present	1	100	–	–
	2	50	–	20
	3	–	40	–
Absent	4	–	10	–
	5	20	–	20
	6	20	–	–
	7	–	–	40
	8	–	–	–
	9	33	–	–

HBV DNA rebound was defined as an increase in serum HBV DNA of greater than or equal to 1 log10 copies/ml from the ADV treatment nadir.

Villeneuve and colleagues[11] also reported a case in which there was selection of the N236T mutant in a post-liver transplantation patient with LMV resistant HBV at ADV treatment month 20. However, selection of the ADV mutation in a large number of LMV resistant CHB patients exposed to ADV has not yet been reported.

A total of 67 LMV resistant CHB patients treated with ADV for a minimum of three months were included in our study. ADV resistance was confirmed by RFMP genotypic analysis and real time PCR quantification of HBV DNA.

A total of 11 ADV mutations occurred in nine of 67 LMV resistant CHB patients during the course of their ADV treatment, and the cumulative incidence of genotypic mutation in our cases at treatment weeks 48 and 96 was 6.4% and 25.4%, respectively, and this was higher than that of a previous report in nucleoside/-tide naïve patients.[8 20] In our study, the first cases of the rtN236T, rtA181V, and rtA181T mutations were detected at treatment months 3, 12, and 7. As previously described,[14] RFMP analysis has the advantage of detecting a ''minor'' population comprising up to 1% of the total viral population.[14 21] In contrast with RFMP, detection of mutation using the PCR sequencing method almost certainly could not have detected the minor species comprising less than 30% of the entire HBV population. In our study, a sensitive and specific RFMP method was used for genotypic mutation analysis. These factors might explain the earlier and higher detection rate of the ADV mutations in our cases.

Eight of nine patients with the ADV mutation had cirrhosis (compensated ($n = 5$) or decompensated ($n = 3$)). Differences in baseline characteristics, including histological severity, that might have contributed to the development of the ADV mutation needs to be studied further.

We selected a definition of HBV DNA rebound as a 1.0 $\log_{10}$ or greater increase in serum HBV DNA level from the treatment nadir. In our study, the cumulative incidence of HBV DNA rebound associated with the ADV mutation was 0 of 67 patients (0%) and three of 67 patients (4.4%) at treatment months 12 and 24, respectively.

In 58 patients with no detectible ADV mutation, serial quantification of HBV DNA revealed HBV DNA rebound in three patients. Westland and colleagues[8] reported that 24 of 294 patients on ADV 10 mg displayed an unconfirmed 1.0 $\log_{10}$ or greater increase in serum HBV DNA levels at week 48 or at the last visit. They suggested that this reflects the fluctuating nature of HBV replication in CHB disease. Although patients showed good compliance to the drug and coinfection with other viruses was not detected, serial quantification and mutation analysis will be able to provide

more information, including the interaction between the previous LMV resistant mutation and ADV.

The clinical course of patients after emergence of the ADV mutation was variable. Although exacerbation of serum bilirubin and serum ALT was observed in two patients, most of the nine patients with the ADV mutation maintained their clinical stability. In our study, two rtA181V mutant patients with or without the rtN236T mutation developed HBV rebound three months after the genotypic mutation, but one patient with the rtA181T mutation developed HBV DNA rebound at the time that the mutant was detected.

In our cases, fractionation of the mutant population by RFMP analysis revealed that the mutant comprised 40–100% of the total population in three patients with HBV DNA rebound. The other cases with no HBV DNA rebound showed the wild-type dominant or transient mutation. As this was observed in a rather small study population, it will be important to confirm these results in a larger cohort of patients.

Angus and colleagues[10] reported that ADV mutation patients did not show any further HBV DNA decrement after an initial log reduction to 2.4, even after six months of therapy. They suggested that antiviral resistance may have begun to emerge towards the end of the first year of therapy. In our study, patients with the ADV mutation showed a significantly lower magnitude of HBV DNA reduction at treatment months 9 and 12 compared with the non-ADV mutation group. Further studies are needed to analyse the importance of the magnitude of HBV DNA reduction for inducing the ADV mutation.

We verified the results of the RFMP analysis using sequence analysis of the HBV polymerase domain. Bartholomeusz and colleagues[22] reported additional possible mutations in the rt domains D, A, and B. According to our sequence analysis data, we could not identify any additional mutations.

The ultimate goal of HBV therapy is to heal hepatic inflammation and necrosis, and thereby halt the progression to cirrhosis and hepatocellular carcinoma. The key to successful therapy for chronic viral hepatitis is to induce durable suppression of viral replication without selection of drug resistant mutations.[23–25] In our LMV resistant CHB patients treated with ADV, emergence of the ADV mutation appeared to present earlier and more frequently than was reported in previous studies in nucleoside/-tide treatment naïve patients. Further studies are needed to define the potential role of the ADV resistant mutations and clinical consequences of those mutations for LMV resistant CHB patients who are treated with long term ADV.

Conflict of interest: declared (the declaration can be viewed on the *Gut* website at http://www.gutjnl.com/supplemental). Supplementary table 1 can also be viewed on the *Gut* website at http://www.gutjnl.com/supplemental

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APPENDIX

Primers used in amplification for the RFMP assays of rtA181V/T and rtN236T are shown in table A1.

Table A1 Primers used in amplification for restriction fragment mass polymorphism (RFMP) assays of rtA181V/T and rtN236T

Primer	Sequences (5′-3′)	Position	Polarity
rfmp181f	CCTATGGGAGTGGGtccaacTCAGGCCGTTTCTC CCTATGGGAGTGGGCtccaacTCAGGCCGTTTCTC	637–666	Sense
rfmp181r	GAAAGCCAAACAGTGGGGGAA AGC	732–709	Antisense
rfmp236f	TTACCAATTTTCTTTTGTCtccaacTGGGTAAATATTT TTACCAATTTTCTTTTGTCTTtccaacGGTAAATATTT TTACCAATTTTCTTTTGTCTT GtccaacGTAAATATTT	800–833	Sense
rfmp236r	TAGCCCCAACGTTTGGTTTTATT	863–841	Antisense

Nucleotide sequence positions were numbered according to Ono and colleagues.[12]
A six nucleotide sequence (tccaac) embedded in the forward primer to introduce a *Mme*I site in amplicon is indicated by small letters.
Sequences modified to introduce *Hae*III(ggcc) and *Ssp*I (aatatt) sites in forward primers are underlined.

EDITOR'S QUIZ: GI SNAPSHOT

Robin Spiller, *Editor*

A case of a ''fragile'' oesophagus

Clinical presentation

A 25 year old man with no allergic history underwent gastroscopy due to longstanding dysphagia. Six months previously he had an episode of food bolus obstruction. He had undergone gastroscopy which had shown no abnormal findings. pH/manometry studies were recommended and were also normal. During intubation and scope insertion, the macroscopic appearance of oesophagus was normal. No strictures were observed and there was no evidence of oesophagitis, Barrett's oesophagus, or hiatus hernia. The rest of the endoscopy was also unremarkable. On withdrawing the endoscope, we noticed a large bleeding mucosal tear at 25 cm from the incisors (fig 1A). Biopsies were obtained from the oesophagogastric junction and mid-oesophagus (fig 1B).

Question

What is the diagnosis?
See page 1511 for answer
This case is submitted by:

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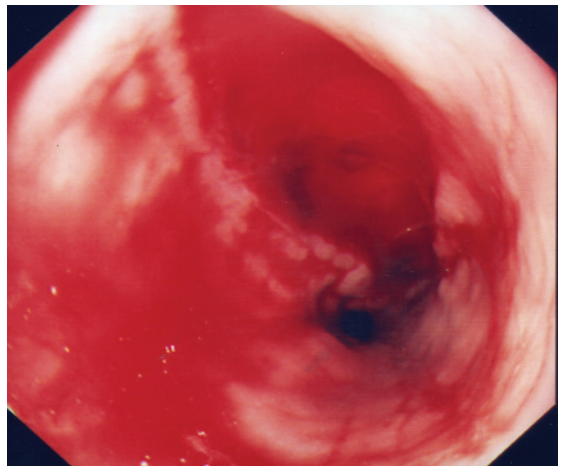

Figure 1 A mucosal tear in the upper-mid oesophagus.

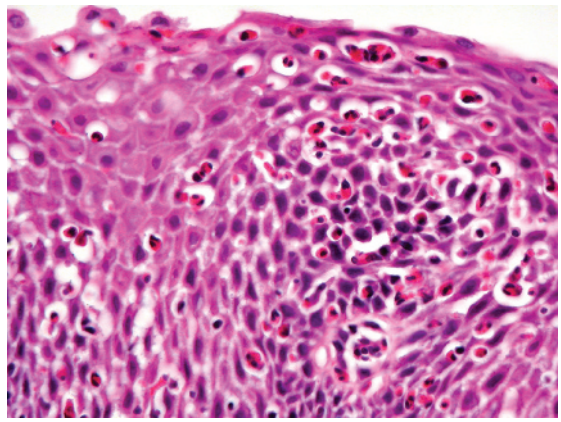

Figure 2 Histology demonstrated eosinophilic infiltration of the oesophagus with >40 eosinophils/high power field.